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Molecular cycloencapsulation augments solubility and improves therapeutic index of brominated noscapine in prostate cancer cells

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Abstract

We have previously shown that a novel microtubule-modulating noscapinoid, EM011 (9-Br-Nos), displays potent anticancer activity by inhibition of cellular proliferation and induction of apoptosis in prostate cancer cells and preclinical mice models. However, physicochemical and cellular barriers encumber the development of viable formulations for future clinical translation. To circumvent these limitations, we have synthesized EM011-cyclodextrin inclusion complexes to improve solubility and enhance therapeutic index of EM011. Phase solubility analysis indicated that EM011 formed a 1:1 stoichiometric complex with β -CD and methyl- β -CD, with a stability constant (K_c) of 2.42×10^{-3} M and 4.85×10^{-3} M, respectively. Fourier transform infrared spectroscopy suggested the penetrance of either O-CH₂ or OCH₃-C₆H₄-OCH₃ moiety of EM011 in the β -CD or methyl- β -CD cavity. In addition, multifarious techniques viz., differential scanning calorimetry, powder X-ray diffraction, scanning electron microscopy, NMR spectroscopy, and computational studies validated the cage complex of EM011 with β -CD and methyl- β -CD. Moreover, rotating frame overhauser enhancement spectroscopy showed that the H_a proton of OCH₃-C₆H₄-OCH₃ moiety was in close proximity with H3 proton of β -CD or methyl- β -CD cavity. Furthermore, we found that the solubility of EM011 in phosphate buffer saline (pH 7.4) was enhanced by ~11 fold and ~21 fold upon complexation with β -CD and methyl- β -CD, respectively. The enhanced dissolution of the drug CD-complexes in aqueous phase remarkably decreased their IC₅₀ to 28.5 μ M (9-Br-Nos- β -CD) and 12.5 μ M (9-Br-Nos-methyl- β -CD) in PC-3 cells compared to free EM011 (~200 μ M). This is the first report to demonstrate the novel construction of cyclodextrin-based nanosupramolecular vehicles for enhanced delivery of EM011 that warrants *in vivo* evaluation for the superior management of prostate cancer.

Keywords

EM011; β -CD; methyl- β -CD; inclusion complexes; dissolution; cytotoxicity; prostate cancer

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Introduction

There is a growing awareness that about 40% of all drugs fail clinical trials due to poor biopharmaceutical properties, such as water insolubility and low bioavailability, and tubulin binding anticancer drugs are no exception. Thus, integration of drug delivery considerations much earlier in drug discovery and development cascade is warranted in order to increase the number of potential candidates available for clinical trials.

Noscapine, a microtubule modulating anticancer drug, is a founding member of the noscapinoid family currently in Phase I/II clinical trials for chemotherapy of multiple myeloma.¹ In an attempt to develop more efficacious analogs based on the phthalideisoquinoline noscapine scaffold, our lab has been continually engaged in rational drug design and chemical synthesis to yield several members of the noscapinoid family.²⁻⁷ One promising molecule, 9-bromonoscapine (9-Br-Nos) also called EM011, is about 10-40 fold more potent and retains the non-toxic features of the parent noscapine.⁸ Extensive literature underscores its preclinical efficacy in various xenograft models including breast, vinblastine- and teniposide- resistant lymphomas, and prostate cancers.⁹⁻¹² However, despite its favorable toxicity profile compared to other tubulin-active drugs, its clinical translation may be limited due to poor dissolution characteristics, sub-optimal pharmacokinetics and low bioavailability in the tumor mass. at the tumor site.

Oral chemotherapy remains a viable option, in particular for the management of chronic diseases such as cancer. The oral route is always preferable by patients for drug administration as it is very convenient, bypasses hospitalizations and thus results in improved compliance. Unfortunately, most currently-available anticancer drugs in particular, the tubulin-binding drugs such as the taxanes and vincas, cannot be administered orally.¹³⁻¹⁴ Unlike taxanes and vincas, noscapinoids do not cause extreme effects on microtubules, thus they are devoid of the harsh toxicities on normal cells.¹⁵ Nonetheless, we can classify noscapine as well as its analog, EM011 as class IV drugs as per the Biopharmaceutical Classification System due to their low solubility and low intestinal permeability.¹⁶ This may also account for the low bioavailability of noscapinoids in systemic circulation after diffusion of the drug from intestinal absorption window.

To circumvent the hurdles imposed by sub-optimal physiochemical characteristics including limited solubility and poor absorption issues of EM011, the FDA approved water-soluble and biocompatible cyclodextrins (CDs) appeared appealing to us for enabling drug solubilization, thus improving overall efficacy. Structurally, CDs are cyclic, bucket-shaped structures consisting of six, seven, or eight glucopyranose units, namely α , β , γ -CD, covalently coupled by α ,1-4-glycosidic bonds to form the macrocycle.¹⁷ Essentially, CDs are enabling excipients employed to address solubility, stability, and bioavailability issues while providing a biocompatible dosage form.¹⁸ β -CD, a fascinating molecule is nontoxic in nature and due to its unique bucket type structure, forms inclusion complexes with a wide array of lipophilic drugs.¹⁹⁻²² However, the limited solubility of β -CD in aqueous phase (18.5 mg/ml) restricts its use in cavitization of lipophilic drugs to form soluble complexes.²³ In addition, the rigid β -CD structure is amenable to crystallization upon complexation. Thus, we also utilized methyl- β -cyclodextrin (methyl- β -CD), a β -CD variant modified through random methylation to produce more wettable amorphous compounds with improved water solubility and complexing power.²⁴ Methyl- β -CD also offers a significant advantage over β -CD as its solubility in aqueous phase is higher than >2,000 mg/ml.²⁵ Thus, to improve the physicochemical and drug delivery characteristics of EM011, we have developed a β -cyclodextrin (β -CD) and methyl- β -cyclodextrin (β -CD) mediated EM011 drug delivery system via the cycloencapsulation technique (Scheme 1). EM011 encapsulation into the CD cavity was accomplished by inclusion complex mechanisms. The formation of β -CD and

methyl- β -CD EM011 complexes were characterized by Fourier transform infrared (FTIR) spectroscopy, differential scanning calorimetry (DSC), powder X ray diffraction (PXRD), scanning electron microscopy (SEM), ^1H NMR and 2D NMR (ROESY) spectroscopy. In addition, we modeled the CD-EM011 complexes *in silico* and executed molecular dynamics simulations to study the dynamics of the complexes and estimate the relative binding affinities. CD-EM011 complexes were evaluated for cytotoxic activity in prostate cancer, PC-3 cells. MTT-based cell proliferation assays demonstrated that β -CD and methyl- β -CD EM011 complexes enhanced EM011 delivery and augmented its pharmacodynamic efficacy in prostate cancer cells compared to EM011 in free-state. Furthermore, the *in vitro* cytotoxicity of the novel inclusion complexes indicated a much higher activity after cycloencapsulation.

Experimental Section

Materials

EM011 ((S)-3-((R)-9-bromo-4-methoxy-6-methyl-5,6,7,8-tetrahydro-[1,3]dioxolo [4,5-g]isoquinolin-5-yl)-6,7-dimethoxyisobenzofuran-1(3H)-one) was synthesized in our laboratory.⁴ Betacyclodextrin (β -CD), Methyl- β -CD, DCl (35 wt % in D_2O , 99 atom% D) and 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) were from Sigma-Aldrich. D_2O (D 99.9%) and dimethyl sulfoxide- D_6 (DMSO-d_6) (D, 99.9% + 1% V/V TMS) were purchased from Cambridge Isotope Laboratories, Inc. NaOD (40 wt % in D_2O , 99+ atom% D) was purchased from Acros Organic. All other chemicals used were of highest analytical grade and used without further purification as provided by the manufacturer.

Cell line and Reagents

Human prostate cancer cell line (PC-3) was maintained in 95% CO_2 atmosphere at 37°C using RPMI supplemented with 10% fetal bovine serum. All experiments were performed with asynchronous cell populations in exponential growth phase (24h after plating).²⁶

Phase solubility analysis

Phase solubility assay was implemented to assign the stoichiometry of drug with cyclodextrins in solution phase.²⁷ Briefly, EM011 (20 mg) was suspended separately in 10 ml of phosphate buffer saline (PBS; pH 7.4) containing β -CD and methyl- β -CD at concentrations ranging from 1-17 mM. Next, samples were stirred in an orbital shaker (200 rpm) for three consecutive days at $37 \pm 1^\circ\text{C}$. After equilibration, the samples were passed through $0.2\ \mu\text{m}$ membrane filter (Millipore, Germany) and the absorbance was read at 298 nm using a UV-Visible spectrophotometer (Beckman Coulter). The apparent stability constant was calculated from the slope of the phase-solubility diagram using Eq. 1:

$$K_c = \text{Slope} / S_o(1 - \text{slope}) \quad (1)$$

Where K_c is the apparent binding/stability constant and S_o is the solubility of drug in the absence of cyclodextrin.

Synthesis of solid inclusion complexes

Solid inclusion complexes of EM011 with CDs were synthesized by mixing EM011 with β -CD and methyl- β -CD separately in aqueous phase (pH 4.5) in 1:1 molar ratio. The resultant solutions were stirred for 24 h in an orbit shaker (200 rpm) at $37 \pm 1^\circ\text{C}$. Subsequently, the solutions were lyophilized and collected as dry samples.²⁸ We also prepared physical mixtures of EM011 with β -CD and methyl- β -CD in 1:1 molar ratio by mixing individual component and passed through sieve #100.

Characterization of solid inclusion complexes

Fourier-transform infrared (FTIR) spectroscopy

We first characterized the solid inclusion complex of EM011 with β -CD and methyl- β -CD employing FTIR spectroscopy. Therefore, spectra of EM011, β -CD, methyl- β -CD, physical mixture of EM011 with β -CD and methyl- β -CD as well as solid inclusion complexes of EM011 with β -CD (9-Br-Nos- β -CD) and methyl- β -CD (9-Br-Nos-methyl- β -CD) (1:1 mM) were recorded using infrared spectrophotometer (Perkin Elmer). KBr was used to prepare sample pellet (2 mg sample/200 mg KBr) at a force of 40 psi for 4 min using a hydrostatic press. The samples were scanned between 450- 4000 cm^{-1} with a resolution of 4 cm^{-1} .

Differential scanning calorimetry (DSC)

DSC was used to confirm the formation of inclusion complexes in the solid state. Characteristic endothermic peaks of EM011, β -CD, methyl- β -CD, physical mixtures of EM011 with β -CD and methyl- β -CD and solid inclusion complexes of EM011 with β -CD (9-Br-Nos- β -CD) and methyl- β -CD (9-Br-Nos-methyl- β -CD) (1:1 mM) were recorded using the differential scanning calorimeter (Mettler-Toledo Thermal Equipment). Nitrogen was used as a carrier gas at the flow rate of 50 ml/min. Thermograms were recorded at a heating rate of 19.99°C/min in the temperature range of 30 to 400°C with 10 mg of sample.

Powder X-ray diffraction pattern (PXRD)

The crystalline configuration of EM011, β -CD, methyl- β -CD, physical mixtures of EM011 with β -CD and methyl- β -CD and inclusion complexes of EM011 with β -CD (9-Br-Nos- β -CD) and methyl- β -CD (9-Br-Nos-methyl- β -CD) were studied using RIGAKU, Rotaflex, RV 200 (Rigaku Corporation, Japan) with Ni filtered, Cu K α -radiation, at a voltage of 60 kV and a current of 45 mA. The scanning rate employed was 2° C/min over the 25°C diffraction angle (2θ) range.

Scanning electron microscopy (SEM)

EM011, β -CD, methyl- β -CD, physical mixtures of EM011 with β -CD and methyl- β -CD as well as solid inclusion complexes of EM011 with β -CD (9-Br-Nos- β -CD) and methyl- β -CD (9-Br-Nos-methyl- β -CD) were examined by scanning electron microscope (SEM) to visualize the surface topography. Samples were prepared by making the film on an aluminum stub. The stubs were then coated with gold to a thickness of 200 to 500 Å under an argon atmosphere using a gold sputter module in a high vacuum evaporator. The coated samples were scanned and photographs were taken with SEM (Jeol-1761, Cambridge, UK).

Nuclear magnetic resonance (^1H NMR) spectroscopy

^1H -NMR spectra were recorded on a BRUKER DPX 300 MHz spectrometer to elucidate the structure of inclusion complexes in solid state. The solution of EM011 (5.0 mM) was prepared in deuterated dimethylsulfoxide (DMSO-d_6). EM011- β -CD complex, EM011-methyl- β -CD, β -CD, and methyl- β -CD (5.0 mM) were prepared separately in deuterated water (D_2O) and then transferred to NMR tubes. The probe temperature was set at 293K. The ^1H NMR spectra were recorded using a simple pulse-acquire sequence (zg30). Typical acquisition parameters were 64 transients, 3.16 kHz spectral window, a 60° pulse angle giving a digital resolution of 0.048 Hz/point, and a 10.4 s acquisition time with relaxation delay of 1.0s. ^1H chemical shifts in D_2O solutions were referenced against 4,4-dimethyl-4-silapentanesulfonate, sodium salt (DSS) with a coaxial inner tube containing 60 μL of 5.0 mM DSS in D_2O . DSS was not used internally to avoid possible interaction with the β -CDs. For DMSO-d_6 solutions, TMS (tetramethylsilane) was used as an internal reference. The 2D ROESY spectra were recorded with a sweep width of 8.5 ppm in both dimensions. The total

ROESY mixing time was set to 150 ms. The spectrum was acquired with 32 scans, 2048 data points in t2 and 512 free induced decays (FIDs) in t1. The data were apodized with a shifted sine-bell square function in both dimensions and processed to a 2×1 K matrix. ROESY data were processed and plotted using Bruker Top Spin 1.3 software. All other NMR data were processed using MestReNova v5.30 for Windows and plotted using Origin 7. Experiments were carried out at $22 \pm 0.5^\circ\text{C}$. The pH values of the D₂O solutions were measured at 25°C using an Orion 720Aplus pH meter calibrated with three buffers of pH 4.0, 7.0, and 10. The pH of the D₂O solutions were adjusted using NaOD/DCl solutions in D₂O.

***In silico* molecular modeling and molecular dynamics simulations**

The initial coordinates of β -CD were obtained from a 2.20 Å resolution X-ray crystal structure (PDB ID 3M3R) for all molecular dynamics simulations and docking studies.²⁹ The starting EM011- β -CD and EM011-methyl- β -CD complexes for the molecular dynamics simulations were obtained by docking EM011 onto β -CD and methyl- β -CD, respectively. AutodockVina³⁰ was used for docking studies and Gauss View 3.09³¹ was used to build EM011. Methyl- β -CD was created from β -CD by adding methyl groups to 2, 3 and 6 positions using Gauss View 3.09 and minimized to remove steric clashes. Gasteiger charges were added to β -CD, methyl- β -CD and EM011 molecules using Autodock ADT. Flexible docking calculations were performed using the following parameters: the grid spacing was 1.0 Å; the box size was 25 Å in each dimension, and the center of β -CD was chosen as the center of the box with large enough space to sample all possible EM011 conformations within the box. Ten binding modes with the lowest binding energies were saved. The conformation with the lowest binding energy was used and assumed to be the best binder. The conformation of the complex with the lowest binding energy was used for molecular dynamics simulations. All simulations were carried out using AMBER 10 suite of programs³² in explicit TIP3P³³ water model using the GLYCAM_06 force field³⁴ for β -CD and methyl- β -CD. The generalized amber force field³⁵(gaff) parameters and bcc charges were used for EM011. The complexes (EM011- β -CD, EM011-methyl- β -CD) were each solvated with TIP3P³³ water model in a periodic cubic box, with the edges of the box at least 10 Å away from any atom of the complex. Each complex was simulated for at least 50 ns, and the first 10 ns were considered as equilibration. During the simulations, an integration time step of 0.002 ps was used to solve the Newton's equation of motion. Particle Mesh Ewald method³⁶ was used to evaluate the long-range electrostatic interaction and a cutoff of 9.0 Å was used for non-bonded interactions. The SHAKE algorithm³⁷ was used to restrain all bonds involving hydrogen atoms. The simulations were carried out at a temperature of 300K and a pressure of 1 bar. The temperature was regulated using the Langevin thermostat with a collision frequency of 1.0 ps^{-1} . The trajectories were saved every 500 steps (1ps). The binding energy was calculated for each conformation generated in the MD simulations using molecular mechanics Poisson-Boltzmann (and generalized Born) surface area (MM-PBSA) method.³⁸ The binding energies were obtained by using MM-PBSA module in AMBER 10. Two methods were used to calculate the electrostatic component of the binding energy for each snapshot of conformations: the more accurate Poisson-Boltzmann (PB) method³⁹ and the less accurate generalize Born (GB) method⁴⁰.

Determination of encapsulation efficiency

The EM011- β -CD and EM011-methyl- β -CD complexes (5 mg) were dissolved separately in 100 ml of PBS (pH 7.4) for calculation of encapsulation efficiency. The EM011- β -CD and EM011-methyl- β -CD inclusion complexes in PBS (pH 7.4) were gently shaken on an orbital shaker for 24h at room temperature. Subsequently, the samples were centrifuged at 15,000 rpm to remove clumps of β -CD and methyl- β -CD respectively. Finally, the respective supernatant solutions containing EM011 were passed through 0.22 μm membrane filters

(Millipore, Germany) and the absorbance was read at 298 nm using a UV-Visible spectrophotometer (Beckman Coulter). The percent encapsulation efficiency was calculated as:

$$\% \text{ Encapsulation efficiency} = \text{Practical value} / \text{Theoretical value} \times 100$$

Determination of solubility in aqueous phase

The solubility of EM011 and inclusion complexes in aqueous phase was determined by preparing the respective saturated solutions. Briefly, 20 mg of EM011 was added to 10 ml of PBS (pH 7.4) and stirred for 24 h in an orbit shaker at $37 \pm 1^\circ\text{C}$. Subsequently the suspensions were centrifuged and supernatants were filtered through $0.22 \mu\text{m}$ membrane filters (Millipore, Germany) and analyzed in a UV-Visible spectrophotometer (Beckman Coulter, USA) at 298 nm. The above procedure was repeated for the inclusion complexes of EM011 with β -CD and methyl- β -CD. All experiments were carried out in triplicates.

In vitro dissolution profile

Dissolution studies were conducted as per the standard protocol using USP dissolution rate test apparatus.⁴¹ We carried out the dissolution study of EM011, physical mixtures and solid inclusion complexes equivalent to 50 mg of drug in 900 ml of simulated intestinal fluid (SIF). A 5 ml sample was withdrawn at 5, 15, 30, 45, 60, 90 and 120 min and equivalent amount of fresh SIF (pH 7.4) was added to mimic infinite sink conditions. The EM011 concentration was determined using UV-Visible spectrophotometer (Beckman Coulter) at 298 nm. The experiment was carried out in triplicate.

In vitro cell proliferation assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was employed to evaluate the proliferative capacity of cells treated with EM011, β -CD, methyl- β -CD and the inclusion complexes.⁴² 5×10^3 PC-3 cells per well were seeded in a 96-well format and after 24h of incubation period, serum RPMI was replaced with serum free RPMI. Next day, cells were treated with EM011, β -CD, methyl- β -CD and inclusion complexes of EM011 with β -CD (9-Br-Nos- β -CD) and methyl- β -CD (9-Br-Nos-methyl- β -CD) at gradient concentrations ranging from 6.25 to 200 μM in PBS (pH 7.4) for 72h. At the end of treatment, 5 mg/ml MTT was added to each well and the plate was incubated at 37°C in dark for 4h. The formazan product was then dissolved by adding 100 μl of DMSO after removing the medium from each well. The absorbance was measured at 570 nm using a plate reader (BioRad).

Statistical analysis

Statistical significance was analyzed with one-way analysis of variance and student *t* test. $p < 0.05$ was taken as a significant level of difference. The results are presented as the mean $\pm$ S.D for $n = 3$.

Results

Characterization of inclusion complexes in solution state

The main focus of the present study was to utilize CD, the biocompatible cyclic oligomers of glucose to enhance the dissolution characteristics, bioavailability and thus the therapeutic index of EM011, a microtubule modulating drug⁴ with a unique mode of action that holds serious promise in non-toxic cancer chemotherapy. In this study, we have explored the supramolecular chemistry approach to stabilize and solubilize EM011 via the lyophilization based cycloencapsulation technique.²⁸ Having synthesized the CD-based solid inclusion

complexes of EM011, our first step was to determine the composition, stoichiometry, and the apparent stability constant (K_c) of these complexes. To this end, we employed phase solubility analysis in solution phase as per the Higuchi and Connors system.²⁷ The phase-solubility diagrams of the complex of EM011 with β -CD and methyl- β -CD in solution state are shown in Figure 1. Both curves demonstrated that the solubility of EM011 increased linearly as a function of concentration of β -CD and methyl- β -CD, respectively. These data allowed us to classify the solubility diagrams of EM011 with β -CD and methyl- β -CD as A_L type.⁴³ The linear host-guest correlation with slope (<1) suggested formation of a 1:1 complex of EM011 with β -CD and methyl- β -CD. The stability constants (K_c) of the binary systems of EM011 with β -CD and methyl- β -CD were calculated to be 2.42×10^{-3} M and 4.85×10^{-3} M respectively, from the linear plots of the phase solubility diagrams.

Characterization of inclusion complexes in solid state

Next, we characterized the inclusion complexes of EM011 with β -CD and methyl- β -CD using FTIR. Spectra were recorded to analyze the alteration of stretching frequencies due to hydrophobic association during the cycloencapsulation of EM011 in β -CD and methyl- β -CD cavity. The assignment of FTIR stretching frequencies of EM011, β -CD, methyl- β -CD, physical mixtures and the inclusion complexes are presented in Table 1. The FTIR spectrum of EM011 showed a characteristic peak at $1,746\text{ cm}^{-1}$ (ν , C=O), indicating the presence of a lactone ring. In addition, drug peaks were observed at $1,033\text{ cm}^{-1}$ and $1,040\text{ cm}^{-1}$ that suggested ortho-substituted benzene. Other peaks observed for EM011 were $2,966\text{ cm}^{-1}$ for C-H stretching vibrations, $1,418$ and $1,380\text{ cm}^{-1}$ for N-CH₃ bending vibrations and $2,950$, $2,890$, $2,839$, $2,815\text{ cm}^{-1}$ due to the presence of various OCH₃/CH₃ groups. The spectrum of β -CD revealed the vibration of free -OH groups at $3,281\text{ cm}^{-1}$ whereas $2,925\text{ cm}^{-1}$ and $1,640\text{ cm}^{-1}$ indicated the presence of -CH stretching and H-O-H bending, respectively. However, the presence of a $2,835\text{ cm}^{-1}$ peak in methyl- β -CD (OCH₃/OCH₂) differentiated it from β -CD. The physical mixture of EM011 with β -CD and methyl- β -CD indicated the presence of identical peaks of individual components. Furthermore, the insertion of EM011 in β -CD and methyl- β -CD cavity by complexation completely masked the characteristic peaks (2839 cm^{-1} and 2815 cm^{-1}). This indicated the absence of OCH₃ or OCH₂ group or insertion of the methoxy group in cyclodextrin cavity. Thus, the FTIR spectra provided us initial information on the functional groups of EM011 which enter the β -CD and methyl- β -CD cavity. However, to confirm the synthesis of inclusion complexes of EM011 with β -CD and methyl- β -CD (9-Br-Nos- β -CD and 9-Br-Nos-methyl- β -CD) in solid state, we used DSC to measure the endothermic peak of inclusion complexes and compared it with individual components as presented in Figure 2. Our data showed that the endothermic peak of EM011 was observed at 183°C close to the melting range of parent compound, nescapine. The thermograms of all CDs (i.e. α -, β and γ -CDs) show a broad range of endothermic peaks ranging from 40 to 150°C (117.83°C for β -CD and 83°C for methyl- β -CD) due to the dehydration process. The thermogram of the physical mixture of EM011 with β -CD and methyl- β -CD indicated the presence of identical peaks of individual components. However, thermograms of inclusion complexes (9-Br-Nos- β -CD and 9-Br-Nos-methyl- β -CD) showed a complete disappearance of the endothermic peaks characteristic of EM011 with a little shift in β -CD and methyl- β -CD endothermic peaks to 149°C and 98°C , respectively.

Our next step was to define the crystal structure of EM011 in the inclusion mode by PXRD technique. Similar to the parent molecule nescapine, the XRD pattern of EM011 showed intense and sharp peaks indicating its crystalline structure (Figure 3 a-g). Although the XRD pattern of β -CD showed sharp peaks indicating its crystalline structure, the induction of methylation in β -CD i.e methyl- β -CD transformed the crystalline structure into an amorphous state that showed undefined broad, diffused peaks. This indicated the improved solubility of methyl- β -CD in water compared to β -CD. The XRD pattern of the physical

mixture of EM011 with β -CD and methyl- β -CD revealed the presence of peaks of both compounds. Finally the inclusion complexes of EM011 with β -CD and methyl- β -CD (9-Br-Nos- β -CD and 9-Br-Nos-methyl- β -CD) showed peaks of diminished intensity. We next employed SEM to visualize the surface texture of the inclusion complexes (Figure 4 a-b). Although this is not a decisive technique to confirm the inclusion complexes, it helps to assess the existence of a single component in the inclusion complex. Consistent with the results of PXRD, EM011 exhibited the presence of regular size crystalline particles. Similarly, we also observed crystalline particles of β -CD but of indefinite shape. The physical mixture of EM011 with β -CD showed the crystalline structure of both compounds and they were also seen adhering to each other. However, the inclusion complex of EM011 with β -CD (9-Br-Nos- β -CD) exhibited particles of narrow size range with a tendency to aggregate, suggesting the existence of amorphous particles. On the contrary, methyl- β -CD did not show the presence of crystalline particles as line native polymer and existed as an amorphous compound. Therefore, the physical mixture of EM011 with methyl- β -CD showed the presence of crystalline as well as amorphous particles, whereas the inclusion complex (9-Br-Nos-methyl- β -CD) assured the existence of only the amorphous product.

We next performed solution phase characterization of the inclusion complexes using ^1H NMR spectroscopy. Essentially, proton NMR can offer information on the free and bound state of a guest molecule based on the differences in the chemical shift. The induced chemical shift, $\Delta\delta$ is defined as the difference in chemical shifts between bound and free guest molecule. Induced shifts were calculated using the following equation: $\Delta\delta = \delta_{(\text{complex})} - \delta_{(\text{free})}$.⁴⁴ Based on this equation, positive and negative signs indicated downfield and upfield shifts, respectively. Figure 5b and 5c show ^1H NMR spectra of free β -CD and methyl- β -CD with respective inclusion complexes in D_2O .

Since H_3 and H_5 protons reside in the interior cavity of β -CD and methyl- β -CD, their signals were shifted upfield most in the presence of the guest molecule, EM011, confirming the formation of an inclusion complex. Additionally, signals for the protons H_1 , H_2 , H_4 and H_6 residing on the outer surface of β -CD and methyl- β -CD were also shifted indicating a conformational change of the host molecule in presence of the guest molecule, as reported in Table 2. Additionally, ^1H - ^1H 2D ROESY experiments were used to validate through-space intermolecular interactions in the CD inclusion complexes.⁴⁵ The interaction of EM011 with β -CD and methyl- β -CD were also demonstrated by using ^1H - ^1H 2D ROESY and are as presented as partial contour plots on Figure 5b and 5c. The correlation between proton H_a of 9-Br-Nos and inner proton H_3 of β -CD and methyl- β -CD has been shown. No significant correlations were detected between the other protons of EM011 and protons of CDs, which confirmed that one of the rings of EM011 was only partially included, as opposed to the deep insertion of the other aromatic rings into the cavity. These findings were consistent with the previously observed $\Delta\delta$ values.

Characterization of inclusion complexes using *in silico* docking and molecular dynamics simulation. To gain further insights, we employed *in silico* docking and molecular dynamics simulation to model the complexation of EM011 with β -CD and methyl- β -CD. Our docking studies revealed that in both complexes (EM011- β -CD and EM011-methyl- β -CD complexes), the $\text{H}_3\text{CO}-\text{C}_6\text{H}_4-\text{OCH}_3$ group of EM011 was inside the β -CD cavity, whereas the Br-attached ring was exposed in solution through the wider rim of β -CD. These initial structures were then used to carry out the molecular dynamics (MD) simulations. The MD simulation of each complex generated at least 40,000 snapshots of conformations. Next, the binding free energy for each snapshot was calculated and a distribution of binding energies was generated. The distributions of the binding free energies between EM011 and β -CD or methyl- β -CD are shown in Figure 6 (b). Qualitatively, both the PB (Poisson-Boltzmann) and the GB (Generalized Born) methods yielded similar results. These results suggested that

EM011 binds more favorably to methyl- β -CD than β -CD. Addition of methyl groups to the primary and secondary hydroxyl groups at 2, 3 and 6 positions in β -CD slightly disrupted the hollow truncated structure due to the loss of hydrogen bond network in upper and lower rims of β -CD. This perhaps causes an increase in the radius of the wider rim (secondary hydroxyl group) and a decrease towards the primary hydroxyl groups (narrower rim). It also decreases the electrostatic potential and increases the hydrophobic nature of the methyl- β -CD cavity (Figure 6c, e). The lower binding energy of EM011 to methyl- β -CD is possibly due to more favorable van der Waals interactions.

The most probable conformation of the EM011- β -CD and EM011-methyl- β -CD complexes are shown in Figure 6 (d, f). These data revealed that EM011 formed a stronger complex with methyl- β -CD than β -CD, with the $\text{H}_3\text{CO}-\text{C}_6\text{H}_4-\text{OCH}_3$ group of EM011 inside the CD cavity. Analysis of encapsulation efficiency, solubility and dissolution profile of inclusion complexes. Having characterized the drug-CD complexes, we next asked if the cyclocomplexation offered enhanced solubility of EM011. We observed significantly ($p < 0.05$) enhanced solubility of the inclusion complexes of EM011 with β -CD (3.8×10^{-3} g/ml) and methyl- β -CD (7.5×10^{-3} g/ml) compared to free EM011 (0.35×10^{-3} g/ml). Thus, in quantitative terms, the solubility of EM011 upon complexation with β -CD and methyl- β -CD increased by ~ 11 and ~ 21 fold, respectively compared to the free drug. We also calculated the encapsulation efficiency of EM011 in EM011- β -CD and EM011-methyl- β -CD inclusion complexes which were observed to be 90.3% and 95.2% respectively. Furthermore, dissolution studies of the developed inclusion complexes of EM011 in simulated intestinal fluid (pH 7.4) presented dissolution curves as shown in Figure 7. These data indicated that only 9.9% of EM011 was released from the hard gelatin capsule at 30 min compared to the EM011 inclusion complex (9-Br-Nos- β -CD) which released 73.9% of drug in the same time interval. Similarly, the dissolution profile of EM011 inclusion complex with methyl- β -CD (9-Br-Nos-methyl- β -CD) released 90.6% of the drug compared to 9-Br-Nos- β -CD complex. However, physical mixtures of EM011 with β -CD and methyl- β -CD did not influence the drug release significantly ($p > 0.05$) as compared to the free drug.

Analysis of *in vitro* cytotoxicity

Finally, we evaluated the cytotoxic activity of EM011, β -CD, methyl- β -CD and the inclusion complexes (9-Br-Nos- β -CD and 9-Br-Nos-methyl- β -CD) in prostate cancer PC-3 cells by dissolving the formulations in phosphate buffer saline (pH 7.4). The cytotoxic activity was evaluated using the standard MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) cell viability assay.⁴² As shown in Figure 8, the lowest IC_{50} value (12.5 μM) was observed for the inclusion complex of EM011 with methyl- β -CD (9-Br-Nos-methyl- β -CD) whereas the β -CD complex (9-Br-Nos- β -CD) exhibited significantly ($p > 0.05$) higher IC_{50} value at 28.5 μM . However, EM011 which was only slightly soluble in phosphate buffer saline (pH 7.4) showed an IC_{50} value of ~ 200 μM in PC-3 cells, which was significantly higher than the complexed forms of EM011.

Discussion

Unlike currently-available tubulin-binding chemotherapeutics, noscapine and its analogs, collectively known as noscapinoids, represent a unique class of microtubule-modulating agents that do not exert extreme effects on microtubules.¹⁵ EM011, a novel member of this family, retains the favorable non-toxic attributes of the parent noscapine, and is significantly more potent than noscapine in anticancer action.⁸ However, its lipophilic character (log P value, 3.03 ± 0.61) and sub-optimal physicochemical properties warrant development of viable formulations for its preclinical development. Therefore, in the present study, we cycloencapsulated EM011 using β -CD and methyl- β -CD to facilitate aqueous solubility and improved the therapeutic index.

Most often, low molecular weight compounds exist in a 1:1 ratio with CD molecule, wherein a single drug molecule is included in the cavity of one CD molecule, with a stability constant of $K_{1:1}^-$ for the equilibrium between the free and associated species.⁴³ As expected, our phase-solubility data suggested that EM011 formed a 1:1 inclusion complex with β -CD and methyl- β -CD in solution phase with low affinity (Figure 1). The phase-solubility diagram can be classified as A_L type showing the formation of a water-soluble complex and suggested a first-order kinetics for the complexation between EM011 and CDs.

Further, various spectroscopic techniques employed to elucidate the structure of the inclusion complex in the solid phase allowed us to confirm the stability of EM011 in the complex. FTIR spectral data showed that EM011 remained stable in the inclusion complex as the lactone ring was intact and was not involved in the complexation. However, the FTIR data suggested that the inclusion mode may be incorporated as either $H_3CO-C_6H_4-OCH_3$ or $O-CH_2-O$ moiety of EM011 in the hydrophobic cavity of CD (Table 1). The DSC thermoanalysis confirmed the formation of a 1:1 inclusion complex in the solid state as the endothermic peak of EM011 disappeared in the inclusion complexes of β -CD and methyl- β -CD when compared with the endothermic peak of β -CD and methyl- β -CD, respectively (Figure 2). Furthermore, PXRD patterns of inclusion complexes of EM011 with β -CD and methyl- β -CD exhibited peaks of diminished intensity in comparison to sharp peaks of EM011 (Figure 3). Thus PXRD spectroscopy substantiated that EM011 resides in the β -CD and methyl- β -CD cavity as polymeric wettable amorphous state. Typically, owing to irregular structural configurations, amorphous phase entails minimal energy thus offer utmost solubility and bioavailability of drugs.⁴⁶

Consistent with the results of PXRD, the photomicrographs of SEM also confirm the existence of EM011 in an amorphous state in β -CD and methyl- β -CD inclusion complexes (Figure 4). We further corroborated our solid state data using 1D, and 2D 1H NMR as well as *in silico* docking studies and molecular dynamics simulations to examine the conformations of EM011 inclusion complexes. Based on the difference in chemical shifts, 1H NMR spectroscopy offers evidence of formation of inclusion complex between guest and host molecules. Essentially, when a guest is incorporated in the host molecule, a significant variation of the microenvironments is known to occur between free and bound states. Chemical shift (δ) of a given nucleus depends on its shielding constant and changes in δ (ppm) values of the host and guest nuclei provide a measure of the degree of complex formation. As the chemical environment of some protons changes upon complexation, there is a consequent variation in the chemical shifts (δ ppm) of 1H NMR resonance (shielding or deshielding effects). Hence, the molecular structure of the inclusion complex of EM011 with β -CD and methyl- β -CD (1:1) was elucidated with 1H NMR and ROESY spectroscopy. ROESY data inferred that H_a proton of $OCH_3-C_6H_4-CH_3O$ entered the β -CD and methyl- β -CD cavity and could be correlated with H_3 proton of the hydrophobic cavity (Figure 5). These data correlated well with our *in silico* molecular modeling (Figure 6). Moreover, the deshielding effect on aromatic protons of EM011 upon complexation suggested that the drug deeply penetrated the host cavity (Table 2).

We observed a significant enhancement of EM011 solubility by ~11 fold and ~21 fold upon complexation with β -CD and methyl- β -CD, respectively. In addition, both complexes exhibited good entrapment efficiency of EM011 in β -CD and methyl- β -CD nanocavities. The dissolution study of inclusion complexes was performed in simulated intestinal fluid and compared with pure compound to explain the improved dissolution profile. Noscaphine and its analogs like EM011 are alkaloid bases with a pK_a of 7.8.⁴⁷

Usually, weakly basic drugs are ionized in stomach pH and stay undissociated at neutral/basic pH and thus are absorbed in to systemic circulation by passive diffusion through the

lipophilic absorption window. The absorption of a weakly basic drug ($pK_a \sim 5-11$) is greatly influenced by change in pH.⁴⁸ EM011, being a weakly basic drug, requires basic physiological dissolution media to remain in a unionized form to facilitate its absorption. We believe that EM011 remains undissociated at neutral/basic pH and incorporation into β -CD and methyl- β -CD macrocycles increases its solubility at intestinal pH. Our data confirmed increased drug dissolution upon complexation with β -CD and methyl- β -CD as the inclusion complexes released significantly ($p < 0.05$) higher amount of drug compared to the pure compound and physical mixtures. This suggested the ready dissolution of EM011 in intestinal media to allow its absorption in systemic circulation. Consistent with the dissolution data, we observed that the inclusion complexes of EM011 with β -CD and methyl- β -CD inhibited the proliferation of PC-3 cells at lower IC_{50} s compared to the free drug. It is likely that the drug-inclusion complexes enhance the rate of diffusion of drug across the cell membrane since EM011 is available in the soluble unionized state in PBS at pH 7.4.

In conclusion, the present study describes the use of FDA approved macrocycles (like β -CD and methyl- β -CD) to enhance the solubility and cytotoxicity of a unique microtubule-modulating non-toxic drug EM011. Using a vast repertoire of spectral techniques and characterization methods and computational studies, our data provide sufficient evidence that the CD-based inclusion complexes improve the physicochemical and biological properties of EM011. These data are encouraging and thus warrant a future in depth *in vivo* study to scale-up the technology for the management of prostate cancer.

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Figure 1.
Phase solubility profile of binary systems of 9-Br-Nos-β-CD and 9-Br-Nos-methyl-β-CD.

Figure 2. Differential scanning calorimetry analysis of 9-Br-Nos, β -CD, physical mixture, 9-Br-Nos- β -CD complex, methyl- β -CD, physical mixture, and 9-Br-Nos-methyl- β -CD complex

Figure 3. PXRD pattern of a) 9-Br-Nos, b) β -CD, c) Physical mixture of 9-Br-Nos and β -CD d), 9-Br-Nos- β -CD complex e) Methyl- β -CD, f) Physical mixture of 9-Br-Nos and methyl- β -CD g) 9-Br-Nos-methyl- β -CD complex

Figure 4. Scanning electron microscopy of a) 9-Br-Nos, β -CD, physical mixture, and 9-Br-Nos- β -CD complex, b) 9-Br-Nos, methyl- β -CD, physical mixture, and 9-Br-Nos-methyl- β -CD complex.

Figure 5.

(a) Structures of EM011 (9-Br-Nos), β -CD and methyl- β -CD with proton numberings related to the ^1H NMR assignment. (b) ^1H 1D spectra of free β -CD and 9-Br-Nos- β -CD complex in D_2O and partial contour plot of the ^1H - ^1H 2D ROESY spectrum of 9-Br-Nos- β -CD complex in D_2O . The correlation between proton H_a of 9-Br-Nos and inner proton H_3 of β -CD has been shown. (c) ^1H 1D spectra of free methyl- β -CD and 9-Br-Nos-methyl- β -CD complex in D_2O and partial contour plot of the ^1H - ^1H 2D ROESY spectrum of 9-Br-Nos-methyl- β -CD complex in D_2O . The correlation between proton H_a of 9-Br-Nos and inner proton H_3 of methyl- β -CD has been shown.

Figure 6. Molecular modeling image of a) 9-Br-Nos b) Calculation of energy of 9-Br-Nos-β-CD complex and 9-Br-Nos-methyl-β-CD complex by GB and PB method c) β-CD d) OCH₃-C₆H₄-CH₃O in-orientation in β-CD cavity e) Methyl-β-CD f) OCH₃-C₆H₄-CH₃O in-orientation in methyl-β-CD cavity.

Figure 7. *In vitro* dissolution profile of 9-Br-Nos, physical mixture 9-Br-Nos and β -CD, 9-Br-Nos- β -CD complex, physical mixture 9-Br-Nos and 9-Br-Nos-methyl- β -CD complex

Figure 8. Percent cell viability of 9-Br-Nos, β -CD, 9-Br-Nos- β -CD complex, methyl- β -CD and 9-Br-Nos-methyl- β -CD complex

Table 1
FTIR spectrum assignment, measured between 4400 to 400 cm⁻¹ of 9-Br-Nos, β -CD, methyl- β -CD, physical mixtures (1:1 for 9-Br-Nos: β -CD and 1:1 for 9-Br-Nos:methyl- β -CD) and 9-Br-Nos- β -CD (1:1) and 9-Br-Nos-methyl- β -CD (1:1) inclusion complexes

Components	Peaks	Assignment
9-Br-Nos ^a	2950 cm ⁻¹ , 2890 cm ⁻¹	
	2839 cm ⁻¹ , 2815 cm ⁻¹	(ν , - OCH ₃ /OCH ₃)
	1746 cm ⁻¹	(ν , lactone ring)
	1418 cm ⁻¹ , 1380 cm ⁻¹	(ν , N-CH ₃)
	1033 cm ⁻¹ , 1040 cm ⁻¹	(ν , Ortho substituted benzene)
β -CD ^b	3281 cm ⁻¹	(-OH stretching)
	2925 cm ⁻¹	(-CH stretching)
	1640 cm ⁻¹	(H-O-H bending)
	1152 cm ⁻¹	(C-H stretching)
	1077 cm ⁻¹	(C-O stretching)
Physical mixture (9-Br-Nos and β -CD)	1022 cm ⁻¹	(C-O-C bending)
	2949 cm ⁻¹ , 2891 cm ⁻¹	(ν , - OCH ₃ /OCH ₂)
	1748 cm ⁻¹	(ν , lactone ring)
	1151 cm ⁻¹	(C-H stretching)
	1022 cm ⁻¹	(C-O-C bending)
Inclusion complex (9-Br-Nos and β -CD)	2918 cm ⁻¹	(-CH stretching)
	1752 cm ⁻¹	(ν , lactone ring)
	1418 cm ⁻¹ , 1380 cm ⁻¹	(ν , N-CH ₃)
	1079 cm ⁻¹	(C-O stretching)
	1024 cm ⁻¹	(C-O-C bending)
Methyl- β -CD ^c	2925 cm ⁻¹	(-CH stretching)
	2835 cm ⁻¹	(ν , - OCH ₃ /OCH ₂)
	1638 cm ⁻¹	(H-O-H bending)
	1154 cm ⁻¹	(C-H stretching)
	1083 cm ⁻¹	(C-O stretching)
Physical mixture (9-Br-Nos and Methyl- β -CD)	1033 cm ⁻¹	(C-O-C bending)
	2935 cm ⁻¹	(ν , - OCH ₃ /OCH ₂)
	1749 cm ⁻¹	(ν , lactone ring)
	1418 cm ⁻¹ , 1380 cm ⁻¹	(ν , N-CH ₃)
	1152 cm ⁻¹	(C-H stretching)
Inclusion complex (9-Br-Nos-Methyl- β -CD)	1024 cm ⁻¹	(C-O-C bending)
	2931 cm ⁻¹ , 2837 cm ⁻¹	(ν , - OCH ₃ /OCH ₂)
	1767 cm ⁻¹	(ν , lactone ring)
	1035 cm ⁻¹	(C-O-C bending)

^a9-Bromonoscaphine,

^bBetacyclodextrin,

^cMethyl-betacyclodextrin

Table 2
Chemical shifts for the protons of β -CD in the free-state and in the complex measured using tetramethyl silane (TMS) as internal standard

Proton	β -CD (ppm)	9-Br-Nos- β -CD (ppm)	δ (ppm)
H ₁ (d)	5.0626	5.0594	-0.0032
H ₂ (dd)	3.6404	3.6387	-0.0017
H ₃ (t)	3.9609	3.9506	-0.0103
H ₄ (t)	3.5774	3.5742	-0.0032
H ₅ (m)	3.8458	3.8389	-0.0069
H ₆ (d)	3.8732	3.8714	-0.0018